Investigation of signal transduction pathways involved in melanoma cell spreading

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Integrins are a major family of heterodimeric adhesion receptors that are responsible for anchoring cells to extracellular matrix and they also can initiate intracellular signal pathways. Here parental and α4-expressing human malignant melanoma cell lines were used to study the effect of protein kinase C (PKC), protein tyrosine kinases (PTKs) and intracellular Ca2+ on α4β1-mediated cell spreading on VCAM-1. Incubation of melanoma cells with PKC inhibitor inhibited α4β1-mediated melanoma cell spreading completely. Effect of intracellular Ca2+ on melanoma cell spreading was also investigated by non-phorbol ester tumor promotor, thapsigargin, which blocks the ability of the endoplasmic reticulum to replenish stocks of calcium which naturally leak out into the cytosol leading to a transient increase in concentration of intracellular calcium. The results showed that α4β1-mediated spreading was also required intracellular calcium involvement. However, in the presence of PTKs inhibitor melanoma cells showed long, thin dendritic projections compared to control cells. Previously, data was obtained from immunofluorescence experiments showed that after genistein treatment, α4-expressing cells exhibited considerable amounts of α4 integrin and PTKs in both the focal contact points as well as over the whole cell. PTKs inhibitor did not have any effect on α4-expressing cells spreading. This could be related to the amount of the PTKs present in these cells.

Integrins are a major family of cell surface receptors that are responsible for anchoring cells to extracellular matrix (ECM) and function as cell-cell adhesion molecules. The integrin family is composed of heterodimers consisting of two non-covalently associated subunits, α and β, both of which are necessary for adhesive binding. The integrin receptor family includes at least 16 distinct α subunits and 8 or more β subunits1 give rise to over twenty different αβ heterodimeric combinations at cell surfaces2. Both the α and β subunit of integrins are membrane glycoproteins with a large extracellular domain, a single transmembrane domain and a short cytoplasmic domain.

Several studies have shown recently that integrins influence a variety of dynamic processes during embryonic development, tissue organisation and inflammation by affecting cell migration, proliferation, differentiation and gene expression1.

These molecules also might serve as targets for the therapy of certain diseases, such as leukocyte adhesion deficiency (LAD), resulting from a structural defect in the β2 subunit3, and Glanzmann’s thrombasthenia, which is a defect or deficiency in αIIbβ3 (ref.4).

Integrins do not serve simply as inert receptors for the ECM but also can interact with cellular signal transduction molecules and transmit signals to the nucleus following stimulation received from interacting with the ECM3-6. These cell adhesion molecules transmit signals from the extracellular space to inside the cell or from inside the cell to the extracellular space by means such as phosphorylation of various proteins, activation of PKC and mobilisation of Ca2+ in the cells in response to integrin-ligand interactions5-10.

Integrin-mediated cell adhesion to ECM components or aggregation of integrin receptors by specific anti-integrin antibodies has been shown to induce tyrosine phosphorylation of a variety of intracellular proteins. For example, in lymphocytes, integrin α4β1 and the T-cell receptor contribute synergistically to tyrosine phosphorylation of PLCγ1.
Ca²⁺ and Mg²⁺ concentrations might regulate the ligand specificity of effective than Mg²⁺ in supporting the adhesion of mediated by the ζα4β1 integrin and VCAM-1, suggesting that physiologically relevant changes in intracellular calcium inhibited migration of these cells and in 3T3 fibroblast cells PKC activity was shown to be necessary for ζα5β1-mediated cell spreading.

Involvement of cations in integrin-mediated cell adhesion has been shown by several groups. For example Mg²⁺ was shown to support adhesion and migration of ζα2β1 to type I collagen in fibroblasts and Mn²⁺ also supported or augmented integrin-independent adhesion, including ζα3β1, ζα5β1, ζα6β4. However the effect of Ca²⁺ was distinct from these two divalent cations. The binding function of ζαβ1 and ζαβ3 to RGD-containing peptides was compared and findings showed that ζαβ3 bound to its ligand, vitronectin, and to short RGD-containing peptides in either Ca²⁺ or Mg²⁺, but ζαβ1 bound to its ligand, fibronectin, and to RGD-containing peptides only in Mg²⁺ and not in the presence of Ca²⁺. These results indicate a regulatory role for Ca²⁺ in integrin function and a possible involvement of the β subunit in cation binding. Ca²⁺ has been shown to be required for migration of neutrophils, since blockade by removal of extracellular calcium or by buffering extracellular calcium inhibited migration of these cells on vitronectin and fibronectin. Ca²⁺ and Mg²⁺ were required in lymphoid cell-endothelial cell adhesion mediated by the ζα4β1 integrin and VCAM-1 (ref.23,24). However, Ca²⁺ was found to be less effective than Mg²⁺ in supporting the adhesion of ζα4β1 to CS1 peptide, but not for adhesion to VCAM-1 suggesting that physiologically relevant changes in Ca²⁺ and Mg²⁺ concentrations might regulate the ligand specificity of ζα4β1 (ref.24).

In this paper, the possible involvement of PKC, PTKs and intracellular Ca²⁺ in ζα4β1-mediated melanoma cell spreading was investigated.

Materials and methods

Cell cultures—Two human melanoma cell lines were used in this paper, HMB2 and ζα4-expressing HMB2-ζα4 human malignant melanoma cell lines are cutaneous melanoma lines, originally HMB2 melanoma cells were derived from metastases.

The cells were cultured in sterile plastic tissue culture flasks in E4 medium supplemented with 10% (v/v) heat inactivated foetal calf serum (GIBCO BRL Paisley, Scotland) and L-glutamine (1mM final concentration) as adherent monolayers. Melanoma cells were incubated at 37°C in 8% CO₂/92% air in the humidified atmosphere provided by an incubator. Cell lines were subcultured at a ratio of approximately 1:5 every five days using trypsin (0.05% (w/v)/EDTA (5mM) to detach cells. After 10-15 passages, new cultures were established from frozen stocks.

The level of ζα4 cell surface expression on HMB2-ζα4 was tested every six months by FACS analysis and they were tested routinely and found to be free of mycoplasma infection.

Antibodies—The monoclonal antibodies which were used in this paper are as followed; anti-ζα4, 7.2 antibody is specific to ζα4 subunit but the function of this antibody is unknown, anti-ζα, P2W7 and anti-ζβ1, 4B7 antibodies are not blocking antibodies. All these antibodies were produced in Imperial Cancer Research Laboratories.

Flow cytometry—The expression of ζα4, ζαv and ζβ1 integrins was assayed by indirect immunofluorescence using a flow cytometric technique. Melanoma cells were harvested with 0.25% trypsin/EDTA, resuspended in complete medium and washed twice with cell wash buffer (0.1% BSA in PBSA). Cell suspensions containing 2x10⁶ cells/50μl were added to each well of 96-well plates and 50μl of control or experimental antibodies also were added to each well. After a 30 min incubation at 4°C, cells were washed three times with cold wash buffer containing FITC-RAM (DAKO). After another 30 min incubation at 4°C, cells were washed with wash buffer four times and resuspended in 10% (v/v) formaldehyde in PBSA. Those preparations analysed the next day were kept at 4°C overnight. Cells were resuspended into 400μl of
PBS and analysed with a flow cytometer (FACScan, Becton Dickinson) using Consort software.

**Adhesion assay**—The interaction of melanoma cells with specific substrates results in the activation of α4β1 integrin. In these experiments [51Cr] was used as an indicator to investigate the rate of adhered melanoma cells. Cell adhesion assays were performed in 96-well plates (Falcon 3912; Becton Dickinson). Wells were coated for 60 min at 37°C, or overnight at 4°C, with 50 μl aliquots of human fibronectin (Sigma Chemicals Ltd., Poole, UK), rsVCAM-1 (British Biotech., UK) or CS-1 peptide diluted with Dulbecco’s PBS. Any potential non-specific cell adhesion sites were blocked for 1 hr at 37°C with 100 μl of 0.1% (w/v) heat-denatured BSA in PBS. The cells were detached with 0.25% trypsin/EDTA, resuspended in 300-400 μl complete medium containing 0.37 MBq [51Cr] and incubated for 45 min at 37°C. After the incubation time, the cells were washed three times with Tris-buffered saline (TBS) pH 7.4 (24 mM Tris/HCl, 137 mM NaCl, 2.7 mM KCl, 0.1% BSA, 2 mM Glucose) and resuspended to a final density of 3 x 10⁵ cell/ml in TBS buffer. Cell suspensions (50 μl) containing 1.5 x 10⁴ cells were added to each well of the 96-well plates and allowed to attach to the substrate for 60 min at 37°C in an incubator maintained with 8% CO₂ in a humidified atmosphere. Non-adherent cells were removed by rinsing the plates in a bath of TBS supplemented with 1 mM CaCl₂ and 0.5 mM MgCl₂. After two washes, the 96-well plates were cut into individual wells and the radioactivity associated with each well determined in a gamma counter (1261 Multigamma; LKB Wallac, Bromma, Sweden). The percentage of adherent cells was determined from a comparison of the residual radioactivity with the total input.

**Calculating results**

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\% \text{ Adhesion} = \frac{\text{Mean cpm of substrate wells} - \text{cpm of BSA wells}}{\text{input (ie cpm assoc. with 50 μl cells)}} \times \% \text{ adhesion}
\]

\[
\text{Standard deviation (SD) of } \% \text{ adhesion} = \frac{\text{SD of mean cpm of substrate wells} - \text{×% adhesion}}{\text{mean cpm of substrate wells}}
\]

**Analysis of melanoma cells with time-lapse cinemicroscopy**—A 6 cm petri dish was coated with VCAM-1 at 10 μg/ml in PBSA overnight at 4°C. 1 ml BSA used as a negative control. The petri dishes were washed three times with PBSA and after the last wash, the petri dishes were blocked with 0.1% BSA in PBSA for 1 hr at 37°C. The cells were trypsinised, rinsed three times in 0.5% BSA in serum free medium containing 1 mM glutamine and resuspended in the same medium. At a density of 1 x 10⁵, the cells were plated into a petri dishes and then visualised directly using an Olympus IMT-2 inverted microscope equipped with phase contrast optics and a CCD video camera (TC2-336P, Fujitsu). Data were captured onto PowerMac 7100 fitted with a 1 Gb hard disk and a 24 bit framegrabber (Neotech imagegrabber, UK). Grafitek Optilab Pro. 2.6.1. software (Grafitek, France) was used for analysis of cell spreading and morphology. The cell size of HMB2-α4 cells in suspension was determined as 305 μM². The area and projection index of the cell were measured using particle area and convex command as described below.

**Spread Index** = The final area of the cell/Original area of the cell

**Projection index** = Convex area — original area/ original area

**Statistical**—Mann-Whitney confidence interval test was used to compare the experimental values.

**Results**

**Expression and binding ability of melanoma cell lines to extracellular proteins**—The α4 subunit was introduced into HMB2 melanoma cells by infection of the cells with a recombinant retrovirus vector (pBabe puro/α4, 2.4 kb) encoding full-length human α4 cDNA which in pBluescript KS⁺ was used as a template to amplify 3.3 kb of the coding region of α4 using the polymerase chain reaction (PCR). pBabe puro/α4 construct was packaged by AM12 fibroblast cells growing in 10 cm petri dishes using the Profection method (Promega). Undiluted virus-containing supernatant obtained from pooled colonies of puromycin-resistant AM12 packaging cells were filtered and then this virus stock was applied to HMB2 cells growing at 60% confluence in 100 mm petri dishes. After 10 days, clones were isolated and placed into selective medium in separate wells of 24 well plates²⁷.

Infected HMB2-α4 cells and parental HMB2 melanoma cells were used to determine the expression and binding ability of α4β1 integrin to the substrates fibronectin, CS1 or VCAM-1.

Expression levels of α4, αv and β1 integrins were determined by Fluorescence-Activated Cell Sorting
(FACS) analysis. HMB2 and HMB2-α4 melanoma cell lines were examined using the following three antibodies: anti-α4 mAb (7.2), anti-αv mAb (P2W7) and anti-β1 mAb (4B7). The determined patterns of integrin expression are shown in Fig.1. Labelling of HMB2-α4 with anti-α4 mAb 7.2 (light blue line) showed that both these cell lines expressed a high level of α4 on their surface. A similar result was obtained with another α4 antibody HP2/1 (data not shown). In contrast, 7.2 did not label HMB2 cells, indicating that these cells do not have any detectable α4 integrin present on their surface. Labelling the above named cell lines with anti-αv mAb, P2W7 (yellow line) showed that the αv integrin was expressed by these cell lines and transferring α4 cDNA into HMB2 melanoma cells did not have any effect on αv integrin expression. Expression levels of β1 were shown to be similar for both parental and transfected cell lines as detected using anti-β1 mAb, 4B7.

The binding ability of these melanoma cell lines was also investigated. ^5^Cr radiolabelled melanoma cells were washed and suspended in Tris buffer and allowed to adhere to the wells of 96-well plates which had been pre-coated with predetermined optimal concentrations of the various substrates. The results, in Fig.2a, show that under these conditions melanoma cell lines bound to fibronectin at between 35% and 40% of the total added. This is consistent with their expression of α5β1, αvβ1 and, in the case of the transfected cell line (HMB2-α4), α4β1 integrin. HMB2-α4 bound to VCAM-1 approximately twice as well as they adhered to CS1 substrates. This may be due to the possibility that VCAM-1 has, unlike CS1, two recognition sites for α4 integrin. In the control cells (HMB2) no significant binding was detected to either of the substrates, VCAM-1 and CS1.

HMB2-α4 and HMB2 melanoma cells were examined to determine whether the binding of melanoma cells to fibronectin, CS1 or VCAM-1 was α4-mediated. The results, shown in Fig. 2b indicate that these cell lines bind to fibronectin (between 20% and 35%) and binding is inhibited by about 30% (P<0.05) in HMB2-α4 cells incubated with anti-α4 monoclonal antibody P2W7. HMB2 cell line was not affected by the presence of this antibody (P= not significant). HMB2-α4 cell line showed a higher percentage binding to VCAM-1 than CS1 which was inhibited completely by mAb P2W7. Another non-α4 mAb (anti-αv, P2W7) had no inhibitory effect on this adhesive behaviour supporting the specificity of mAb P2W7's inhibitory effect. This also supporting by other works where mAb P2W7 was shown to block the epitope responsible for interaction of α4 with both VCAM-1 and fibronectin.

Effect of PKC on melanoma cell spreading—The effect of PKC on melanoma cell spreading was examined using the specific PKC inhibitor, Calphostin C (50nM), by monitoring its effects by

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![Fluorescence Intensity Graph](image-url)

Fig. 1—Indirect immunofluorescence staining with anti-α4 mAb (7.2), anti-αv mAb (P2W7) and anti-β1 mAb (4B7). HMB2 and HMB2-α4 melanoma cell lines were stained with these antibodies for 30 min on ice followed by RAM-FITC.
Fig. 2—The adhesion of HMB2-α4 and HMB2 melanoma cell lines to various extracellular proteins. Binding was inhibited by the α4-blocking antibody HP2/1. Panel a, HMB2 (α4-negative) and HMB2-α4 (α4-positive) cells were suspended in Tris-HCl buffer. The cells were placed into the wells of a 96-well plate coated with 10μg/ml fibronectin, 20μg/ml CS1 or 20μg/ml VCAM-1. The cells were allowed to adhere for 1 hr at 30°C. Panel b, Cells were plated into wells containing 10μg/ml Hrp2/1 and incubated at 4°C for 10 min and then plates were transferred to 30°C for further incubation. Cell adhesion was quantified as described in Materials and Methods. The data represent means±SD from triplicate wells.

'Time-Lapse' microscopy. Two parameters were chosen to describe cell spreading. First the ability of cells to spread and second the index of the induced cell projections. Adhesion assay showed that HMB2-α4 cells adhered to VCAM-1 via α4β1 integrin unlike HMB2 cells which do not express α4 integrin did not bind to VCAM-1 coated plates (Fig.2). Fig.3c showed that untreated HMB2-α4 cells (control) were fully spread after a 2hr incubation at 37°C and that, if anything, the level of spreading decreased slightly between 2 and 12hr. Similarly, the index of the cell projections (Fig.3b) attained a maximum over a 4hr period which was sustained during a further 8hr of incubation. By comparison HMB2-α4 cells incubated with 50nM Calphostin C for 1hr in light showed a reduced ability to spread (67% of the control) (Fig.3c) and a greatly reduced index of cell projection (80% of the control) (Fig.3b) when incubated on 10μg/ml VCAM-1. In Fig.3a, the spreading index was shown as a μm².

Figures 4 and 5 show the morphological changes between untreated and Calphostin C-treated HMB2-α4 cells as a function of time. As described above untreated HMB2-α4 melanoma cells started to spread within 1hr. Routinely, they were maximally spread and showed long, thin projections at 12hr. However as shown in Fig. 5 when HMB2-α4 cells were treated with 50nM Calphostin C, spreading of this cell line on VCAM-1 was blocked completely even after 12hr; strongly supporting the view that PKC activity is required for melanoma cell spreading.

Effect of protein tyrosine kinases on melanoma cell morphology—The effects of protein tyrosine kinases on melanoma cell surface area, the index of cell spreading and the index of cellular projections were investigated in HMB2-α4 cells using the protein tyrosine kinase inhibitor Genistein. Untreated HMB2-α4 cells spread rapidly, attaining a maximum value 1-2hr after plating (Fig.6c) and this was reflected in the calculated spread index. Treatment with Genistein had little or no discernible effect upon these parameters (Fig.6c). Marked morphological changes were apparent following inhibitor treatment and these were manifested as increased dendritic projections and a steadily increasing projection index over the 12hr observation period; resulting in a 3-4 fold increase in this parameter. The figures represent the mean of 30 individual cells. These long, thin dendritic processes are very evident in the photographs presented in Fig.7.
Effect of intracellular Ca\(^{2+}\) on melanoma cell morphology—The effect of thapsigargin on melanoma cell (HMB2-α4) spreading and the index of cellular projections was investigated by using Time-Lapse analysis. Fig. 8, shows control HMB2-α4 melanoma cells on VCAM-1. The spreading index (Fig. 8c) of control HMB2-α4 cells on VCAM-1 was 1.1 at the 0 time point and increased to a maximal 6.2 by 2 hr. After 4 hr incubation the HMB2-α4 cells showed a slight decrease in spreading index on VCAM-1 but then the level of spreading index remained constant over an 8 and 12 hr incubation period (4.5). However, the treatment of HMB2-α4 cells with 100 nM thapsigargin for 30 min decreased

Fig. 3—Effect of PKC inhibition on melanoma cells spreading. Untreated HMB2-α4 cells are indicated by black rectangle (■) and HMB2-α4 treated with 50 nM PKC inhibitor, Calphostin C for 1 hr are showed by striped rectangle (■■). The cells were washed and plated onto 6 cm dishes coated with 10 μg/ml VCAM-1. In both cases cells were analysed by Time-Lapse using Graftek Optilab Pro 2.61 software. Data shown are derived from three independent experiments.

HMB2-α4 melanoma cells were harvested with trypsin/EDTA and washed thrice with E4 medium containing 0.5% BSA. Density of 5×10⁴ cells/ml was placed on 6 cm petri dishes coated with VCAM-1 at 4°C overnight. Experiment was carried out at 37°C and cells were gassed with 5% CO₂. Images were captured every minute by Time-Lapse and these photographs derived from these records. Magnification bar=50μM.
the spreading ability on VCAM-1 as compared to control HMB2-α4 cells (Fig.8c). This decrease, which was significant meant that thapsigargin treated HMB2-α4 cells showed a 1.1 spreading index at the 0 time point and then went up to a maximal level at 4hr of 2.3. After 4hr incubation, the level of spreading index remained unchanged at 2.2. Depletion of intracellular calcium stores also had an effect on the index of melanoma cell projections (Fig.8b). Thus the control HMB2-α4 cells showed an average index of cell projections which was 0.3 at 1hr and which increased to a maximal 0.7 at 4 hr and then showed a

![Fig. 5—Effect of calphostin C on melanoma cell morphology. HMB2-α4 melanoma cells were harvested with trypsin/EDTA and washed 3 times with E4 medium containing 0.5% BSA. 50nM Calphostin C was added to cells and incubated with light for 1 hr. Excess of calphostin C was washed off with medium and then 5x10⁶ cells/ml were placed on 6 cm petri dishes coated with 10 μg/ml VCAM-1. Images were captured every minute by Time-Lapse. Magnification bar=50μM](image)

![Fig. 6—Effect of PTKs on melanoma cell spreading. Untreated HMB2-α4 cells are indicated black rectangle (■) and HMB2-α4 treated with 50μM genistein for 1 hr showed by striped rectangle (□). Cells were plated onto 6 cm dishes coated with 10μg/ml VCAM-1. In both experiments, cells were analysed by Time-Lapse using Graftek Optilab Pro. 2.6.1 software. Data shown are derived from three independent experiments.](image)

![Fig. 8—Effect of thapsigargin on melanoma cell spreading. HMB2-α4 melanoma cells are showed by black rectangle (■) and HMB2-α4 treated with 100 nM thapsigargin for 30 min are showed by striped rectangle (□). Data represent results from three independent experiments.](image)
small decrease to 0.5 at 8 hr. However thapsigargin-treated HMB2-α4 melanoma cells exhibited cell projection index which increased to a maximal 0.45 at 8 hr and showed a small reduction after 12 hr (0.41).
The morphological appearance of HMB2-α4 melanoma cells treated with 100nM thapsigargin, taken at various time points after initiation of treatment, are presented in Fig. 9.

Discussion
Examination of these two melanoma cell lines showed that α4β1 is expressed only HMB2-α4 melanoma cell line established from populations which were infected with a recombinant retrovirus vector encoding the full-length human α4 cDNA. Whereas HMB2 cells do not express α4 integrin on their cell surface. α4β1 integrin is also commonly expressed on other human melanoma cell lines such as DX3, A375, SK23 and MeWo.
Inhibition of PKC by PKC inhibitor, 50nM Calphostin C showed that the spreading of HMB2-α4 melanoma cells to VCAM-1 was inhibited significantly. PKC activity seems to be required for α4β1-mediated cell spreading in HMB2-α4 cells. Therefore activation of PKC might be one of the mechanism to prevent motility of melanoma cells.

A number of reports have also shown that activation of PKC was required for cell adhesion and spreading in other cell types. For example, inhibition of PKC in 3T3 cells plated on fibronectin blocked cell spreading as well as FAK activation, while the production of DAG followed by translocation of PKC was induced in HeLa cells when cells were adhered to collagen through collagen receptors. In human melanoma cells, α3 and β1-mediated adhesion was enhanced by PKC activation.

On the other hand, HMB2-α4 melanoma cells when plated on VCAM-1 did not show any inhibition of spreading when the assays were performed in the presence of PTKs inhibitor, 50μM Genistein. The subcellular distribution of PTKs in these cells, plated on VCAM-1, were examined by immunofluorescence techniques and the results showed that a large amount of PTKs in HMB2-α4 cells accumulated in the focal contacts. This finding may suggest a possible reason for the strong binding of HMB2-α4 cells to VCAM-1 in the response to PTKs inhibitor. The concentration of PTKs inhibitor used in the spreading assays might not be sufficient to inhibit PTKs in HMB2-α4 cells plated on VCAM-1. Alternatively or perhaps additionally, the activation of more than one signalling pathway might be required for HMB2-α4 cell spreading to VCAM-1. Using combinations or individual specific inhibitors for the possible signal transduction molecules, which are involved in HMB2-α4 cells spreading to VCAM-1.

Integrins-mediated cell adhesion and spreading has been shown to induce phosphorylation on tyrosine residues of a number of proteins, such as FAK. Thus, for example the α5β1 integrin mediating attachment to fibronectin in NIH 3T3 cells or α2β1 to collagen in platelets, the αIIbβ3 integrin controlling binding to fibrinogen in platelets all have been shown to induce this event. Equally the α4β1 integrin by adhesion to fibronectin in CHO cells or by antibody cross-linking in T and B lymphocytes has evoked similar changes.

Several studies have shown that integrins require one or more cations to mediate adhesion to their ligands. For example, αvβ3-mediated adhesion of glioblastoma cells to RGD-containing peptides and to vitronectin was enhanced in the presence of Ca2+ (ref.21). However the α2β1-mediated adhesion of platelets to collagen was reduced by the addition of Ca2+ (ref. 37) suggesting that the response to this cation is attributable, at least in part, to the identity of the integrin receptor.
The result shown here suggested that intracellular Ca\textsuperscript{2+} indeed was required to mediate HMB2-\(\alpha_4\) cell spreading on VCAM-1.

Taken together, \(\alpha_4\beta_1\)-mediated melanoma cells spreading on VCAM-1 required activation of PKC and intracellular calcium but the role of PTKs seems to be more complicated and that needs to be investigated in more details.

**References**

18. Grzesiak J J, Davis G E, Kirchhofer D & Pierschbacher M D, Regulation of \(\alpha_2\beta_1\) mediated fibroblast motility on type I collagen by shifts in the concentrations of extracellular Mg\textsuperscript{2+} and Ca\textsuperscript{2+}, *J Cell Biol*, 117 (1992) 1109.


37 Santoro S A, Identification of a 160,000 dalton platelet membrane-protein that mediates the initial divalent cation-dependent adhesion of platelets to collagen, Cell, 46 (1986) 913.